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Advances in Brief

Gefitinib Induces Apoptosis in the EGFR^{L858R} Non–Small-Cell Lung Cancer Cell Line H3255

Sean Tracy,[1] Toru Mukohara,[1,2,3] Mark Hansen,[4] Matthew Meyerson,[1,2,5,6,7] Bruce E. Johnson,[1,2,3] and Pasi A. Jänne[1,2,3]

[1]Lowe Center for Thoracic Oncology and [2]Department of Medical Oncology, Dana Farber Cancer Institute; [3]Department of Medicine, Brigham and Women's Hospital and Harvard Medical School; [4]Shannon McCormack Advanced Molecular Diagnostic Laboratory, Dana Farber Cancer Institute; and Departments of [5]Pathology, [6]Biological Chemistry, and [7]Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts

Abstract

Somatic mutations in the tyrosine kinase domain of the epidermal growth factor receptor (EGFR) have recently been described in patients with non–small-cell lung cancer (NSCLC) who achieve radiographic regressions to the EGFR inhibitor gefitinib. One of these mutations, L858R (Leu→Arg), is also found in NSCLC cell line H3255, which is very sensitive to gefitinib treatment. We characterized nine NSCLC cell lines (three isolated from patients with bronchioloalveolar carcinoma and six isolated from patients with adenocarcinoma) for their *in vitro* sensitivity to gefitinib. Of these, only H3255 (EGFR^{L858R}) and H1666 (EGFR^{WT}) are sensitive to gefitinib with IC_{50} values of 40 nmol/L and 2 μmol/L, respectively. We examined the effects of gefitinib on H3255 and cell lines containing wild-type EGFR that are either sensitive (H1666) or resistant (A549 and H441) to gefitinib exposure *in vitro*. Gefitinib treatment (1 μmol/L) leads to significant apoptosis accompanied by increased poly-(ADP-ribose) polymerase cleavage only in the H3255 cell line, leads to G_1-S arrest in H1666, and has no effects in the A549 and H441 cell lines. Although EGFR and AKT are constitutively phosphorylated in H3255, H1666, and H441 cell lines, AKT is completely inhibited by gefitinib treatment only in the H3255 cell line. These findings further characterize a mechanism by which gefitinib treatment of NSCLC harboring EGFR^{L858R} leads to a dramatic response to gefitinib.

Introduction

The epidermal growth factor receptor (EGFR) is a member of the receptor tyrosine kinase family, which includes the erbB family. EGFR is frequently overexpressed in non–small-cell lung cancer (NSCLC) and has been implicated in the pathogenesis of this disease (1). Clinical responses to gefitinib (ZD1839 or Iressa; AstraZeneca, Waltham, MA), a small molecule EGFR tyrosine kinase inhibitor, in NSCLC have varied among populations, with higher rates of response seen in females, nonsmokers, and those patients with adenocarcinoma or bronchioloalveolar carcinoma (BAC) histology (2, 3). However, clinical responses do not correlate with tumor expression of EGFR (4).

We and others have recently uncovered somatic mutations in the tyrosine kinase domain of EGFR in patients who achieve significant clinical responses to gefitinib (5, 6). These mutations include both small deletions and missense mutations. One of these, L858R (Leu→Arg), occurs in a highly conserved amino acid among protein kinases and is found both in patients who develop significant clinical regressions to gefitinib and in adenocarcinoma cell line H3255, which is hypersensitive to gefitinib *in vitro*. The mechanism through which gefitinib treatment of EGFR^{L858R} leads to tumor regression is presently unknown.

Materials and Methods

Cell Culture. Three BAC [NCI-H358, NCI-H1666, and NCI-H1781 (referred to henceforth as H358, H1666, and H1781, respectively)] and six adenocarcinoma cell lines [NCI-A549, NCI-H23, NCI-H441, NCI-H2347, NCI-H3122, and NCI-H3255 (referred to henceforth as A549, H23, H441, H2347, H3122, and H3255, respectively)] were used in this study and were either purchased from American Type Culture Collection or obtained from the National Cancer Institute and have been characterized previously (5, 7). Cell lines were maintained as described previously (5).

Drugs. Gefitinib was a gift of AstraZeneca. Stock solutions were prepared in dimethyl sulfoxide and stored at −20°C. The drugs were diluted in fresh media before each experiment, and the final dimethyl sulfoxide concentration was <0.1%. Epidermal growth factor (EGF) was purchased from Biosource International Inc. (Camarillo, CA).

Growth Inhibition Assay. Growth inhibition was assessed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)2H-tetrazolium, inner salt) assay (Promega, Madison, WI). These studies were performed according to methods established previously in our laboratory (5). The number of cells per well used in these experiments for the cell lines were as follows: H1781, 15,000; H1666, 1,500; H441, 2500; H3255, 7500; H23, 3000; H2347, 7500; H3122, 5000; H358, 7500; and A549, 1500.

Antibodies and Western Blotting. Cells were lysed in buffer containing proteinase inhibitors, and proteins were separated by gel electrophoresis on 5% to 12% polyacrylamide gels selected depending on the molecular weight of the target, transferred to nitrocellulose membranes and detected by immunoblotting using an enhanced chemiluminescence system (5). EGFR (SC-03) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The phospho-specific EGFR (pY1068), extracellular signal-regulated kinase (ERK) 1/2, and phospho-ERK (pT185/pY187) antibodies were purchased from Biosource International Inc. The phospho-specific Akt (pS473) and total Akt and poly(ADP-ribose) polymerase (PARP) antibodies were obtained from Cell Signaling Technology (Beverley, MA). α-Tubulin antibody was purchased from Sigma-Aldrich (St. Louis, MO).

Cell Cycle Analysis. Cells were plated at a density of 0.5 to 2 × 10^5 cells per plate in 10-cm^2 plates (Becton Dickinson, Franklin Lakes, NJ). Gefitinib was added to the medium after 24 hours, and the cells were incubated for another 72 hours, after which the cells were analyzed as described previously (8). The percentage of apoptosis was estimated from the sub-G_1 cell fraction.

Epidermal Growth Factor Receptor and K-ras Sequencing. Total RNA was isolated from all NSCLC cell lines using Trizol (Invitrogen, Carlsbad, CA) and purified using RNeasy mini-elute cleanup kit (Qiagen, Valencia, CA). Complementary DNA was transcribed from 2 μg of total RNA with Superscript II reverse transcriptase (Invitrogen Life Technologies, Carlsbad, CA). The cDNA was used as template for subsequent polymerase chain reaction (PCR) amplifications of EGFR. The details of the PCR conditions and the primers have been published previously (5).

K-ras was sequenced from the A549, H1666, and H3255 cell lines using cDNA and genomic DNA. The sequencing was designed to cover codons 12,

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Note: S. Tracy and T. Mukohara contributed equally to this work.

Requests for reprints: Bruce E. Johnson, Lowe Center for Thoracic Oncology, Dana-Farber Cancer Institute, D1234; 44 Binney Street, Boston, MA 02115. Phone: 617-632-4790; Fax: 617-632-5786; E-mail: bejohnson@partners.org.

13, and 61. The PCR conditions and primer sequences are available on request. The PCR products were sequenced according to the manufacturer's recommendations and as described previously (5). H441 is known to contain a codon 12 K-ras mutation.

Transforming Growth Factor α Detection Assay. Transforming growth factor (TGF)-α determination in the media of NSCLC cells was performed similarly to a previously described method for detecting hepatocyte growth factor (9). For the determination of TGF-α, enzyme-linked immunosorbent assays were performed according to the manufacturer's recommended procedures (human TGF-α immunoassay; Quantikine; R&D Systems, Minneapolis, MN). All samples were run in triplicate.

Quantitative Polymerase Chain Reaction. Quantitative genomic PCR was used to determine EGFR copy number. DNA was prepared from each of the nine cell lines and from the peripheral blood of five normal volunteers using Qiagen genomic tips. All quantitative PCR reactions were carried out in an ABI 7700 thermal cycler using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA). The PCR conditions are available on request. The amount of template calculated using the *EGFR* probe was divided by the average amount of template calculated from seven reference genes distributed throughout the genome (*CD20*, 11q13; *FANCA*, 16q24; *GART*, 21q22; *MAPK4*, 18q12-q21; *TSN*, 2q21; *VEST1*, 8q13; and *FLT3*, 13q12) to generate the relative *EGFR* copy number. The average relative *EGFR* copy number for the five normal specimens was 2.54 (range, 2.12–2.92). For the nine tumor samples, the average relative *EGFR* copy number was 5.14 (range, 1.68–22.98). The PCR primers for EGFR were derived from exon 16 and are available for all genes on request.

Results and Discussion

Gefitinib Inhibits Growth of Non–Small-Cell Lung Cancer Cell Lines. The effects of gefitinib on growth of NSCLC lines were determined by MTS assay. NSCLC cells were treated with increasing concentrations (3.3 nmol/L to 10 μmol/L) of gefitinib for a duration of 72 hours. Gefitinib achieved a 50% growth inhibition (IC_{50}) at concentrations of <10 μmol/L in two of nine (22%) cell lines, the female-derived BAC cell line H1666 at 2.0 μmol/L, and the female-derived adenocarcinoma cell line H3255 at 40 nmol/L. In the other cell lines, gefitinib achieved various inhibitions ranging from 0% to 48%; all had IC_{50} values of >10 μmol/L. There was no clear relationship between the amount of phospho-EGFR or EGFR and sensitivity to gefitinib (Fig. 1). We sequenced EGFR in all of these cell lines; four of which have been published previously (Table 1; ref. 5). Only H3255 contained a mutation in EGFR (L858R). The strategy of enriching the group of cell lines under study to mirror the clinical population most responsive to gefitinib identified two sensitive cell lines. Both of the sensitive cell lines are also derived from women who are nonsmokers. This frequency is similar to the radiographic response rate observed after gefitinib treatment in this clinically enriched patient population (3). We chose to further examine four cell lines: A549, H441 (both EGFR^{WT} and gefitinib resistant), H1666 (EGFR^{WT}; gefitinib sensitive), and H3255 (EGFR^{L858R}; gefitinib sensitive). A549 was chosen because its response to gefitinib has been extensively characterized (10).

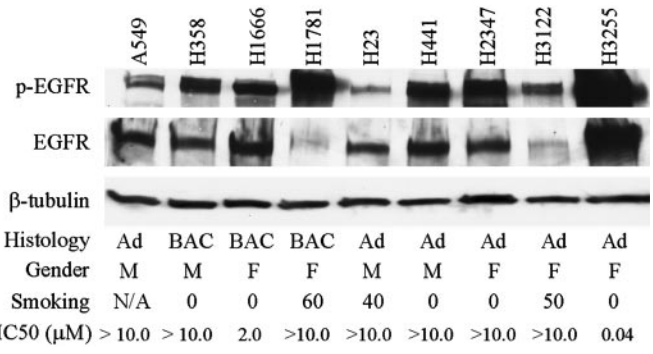

Fig. 1. Cell line characteristics. Western blots for phospho-EGFR and EGFR are shown for the nine cell lines used in this study. The blot has been stripped and reprobed with α-tubulin as a loading control. Shown are the histologies, gender, smoking status (in pack-years), and IC_{50} for growth inhibition by gefitinib. *Ad*, adenocarcinoma; *F*, female; *M*, male; *NA*, not available.

Table 1 *EGFR mutation and relative EGFR copy number in NSCLC cell lines*

Cell line	EGFR mutation	Relative EGFR copy number
A549	None	2.48
H23	None	2.76
H358	None	2.82
H441	None	1.68
H1666	None	2.58
H1781	None	3.96
H2347	None	4.18
H3122	None	3.10
H3255	L858R	22.98

EGFR^{L858R} Is Amplified in H3255. Western blotting (Fig. 1) with anti-EGFR antibodies demonstrated a stronger signal from H3255 than from the other cell lines and suggested that EGFR may be amplified in this cell line. To determine EGFR copy number, we performed quantitative PCR for EGFR and seven reference genes distributed throughout the genome. The relative EGFR copy number for each cell line is shown in Table 1. EGFR is amplified approximately 11-fold in H3255, whereas there is no significant copy number change in the remaining cell lines. On analysis of the sequencing peaks from this cell line at bp 2573 (site of the L858R mutation; CTG→CGG), there was very little wild-type allele (T) compared with the mutant allele (G; data not shown; ref. 5). This suggests that the L858R allele is preferentially amplified in H3255. Gene amplification of EGFR has been noted using fluorescence *in situ* hybridization in 9% of NSCLC but does not appear to correlate with any particular histologic subtype (11). It is presently not known whether EGFR amplification correlates with tumor regression in patients with NSCLC receiving gefitinib, although EGFR expression as detected by immunohistochemistry does not correlate with antitumor response to gefitinib (4). EGFR amplifications have not been previously correlated with EGFR mutations in NSCLC tumor specimens. In breast cancer in which Her2/neu (erbB2) is frequently amplified, the antitumor response to trastuzumab, a Her2/neu-specific antibody, is limited to patients whose tumors have 3+ amplification by fluorescence *in situ* hybridization (12, 13). The epidermoid carcinoma cell line A431, which is very sensitive to gefitinib *in vitro*, is known to contain an EGFR amplification but is not known to contain a mutation in EGFR (14). Thus it is possible that EGFR amplification in addition to mutation in EGFR in H3255 contributes to its sensitivity to gefitinib. In a recent study, Lynch et al. (6) found that gefitinib treatment caused greater growth inhibition in Cos-7 cells containing EGFR^{L858R} expression constructs than in those expressing EGFR^{WT}, suggesting that the L858R mutation contributes at least in part to the sensitivity to gefitinib.

Gefitinib Induces Apoptosis in H3255 Cell Lines. To examine the mechanism of growth inhibition of gefitinib in H1666 and H3255 cell lines, we performed cell cycle analyses. All cell lines were treated for 72 hours in the presence and absence of 1 μmol/L gefitinib. We chose 1 μmol/L because this is a concentration of gefitinib that can be achieved in serum in patients being treated with gefitinib and also one used by other investigators, allowing us to compare our findings (10, 15). As can be seen, only in the H3255 cell line does gefitinib induce a substantial fraction of apoptosis (Fig. 2*A*). The mean percentage of apoptosis increases from 2.14 $\pm$ 0.91% (untreated) to 24.73 $\pm$ 1.85% (1 μmol/L gefitinib). These differences are statistically significant ($P = 0.034$; paired t test). A similar degree of apoptosis was also observed in the H3255 cell line even with 100 nmol/L gefitinib

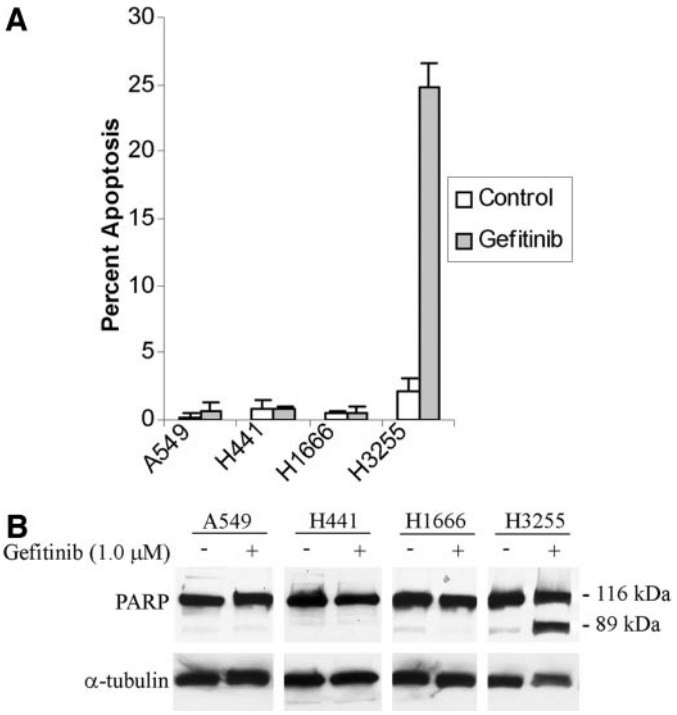

Fig. 2. *A*, quantification of apoptosis. NSCLC cells were grown in the absence or presence of 1 μmol/L gefitinib for 72 hours. Cell cycle analysis was performed as described in Materials and Methods. Shown are the mean percentage of apoptosis and SD from three independent experiments. *B*, Western blot for PARP with NSCLC cell lines grown in the absence or presence of 1 μmol/L gefitinib. The cleaved 89-kDa fragment increases substantially with gefitinib treatment only in H3255 cells. The blot was stripped and reprobed with α-tubulin as a loading control.

treatment (data not shown). The H1666 cell line undergoes cell cycle arrest at the G_1-S boundary after gefitinib treatment [absolute change (mean $\pm$ SD) in G_0-G_1 and S fractions, 14.2 $\pm$ 3.55% and $-9.81 \pm 2.43\%$, respectively], consistent with multiple prior observations using EGFR inhibitors across tumor types including lung cancer (10, 16, 17). There was no significant effect on cell cycle progression in A549 or H441 cells treated with gefitinib (data not shown), consistent with previously published studies (10). No apoptosis was observed in H1666, A549, or H441, even with 10 μmol/L gefitinib treatment (data not shown). We also performed Western blotting for PARP cleavage, an indicator of caspase-mediated apoptosis, after gefitinib treatment. The cleaved 89-kDa fragment increases substantially only in the H3255 cell line after gefitinib treatment (Fig. 2*B*). These findings suggest a critical role for the mutant EGFR receptor for cell survival in H3255 cells. Furthermore, they are consistent with the observations that only a minority of patients with NSCLC, those with EGFR mutations, treated with gefitinib have substantial antitumor responses. H1666, although it contains EGFR^{WT}, undergoes cell cycle arrest after gefitinib treatment. This observation may be an *in vitro* equivalent of stable disease also observed in a substantial fraction of patients who are treated with EGFR inhibitors (2). Additional studies including sequencing of EGFR from tumors of such patients will need to be performed to validate this hypothesis.

Epidermal Growth Factor Receptor Is Constitutively Phosphorylated in Gefitinib-Sensitive Non–Small-Cell Lung Cancer Cells. To determine whether the L858R mutation leads to constitutive activation of EGFR, we examined EGFR phosphorylation in serum-starved conditions with and without gefitinib and EGF treatment. In serum-starved H3255 cells, EGFR is highly phosphorylated and can be further phosphorylated with the addition of exogenous EGF (Fig. 3). In contrast, there is no EGFR phosphorylation in A549 cells (EGFR^{WT}), except with the addition of EGF. Gefitinib treatment for 2 hours substantially decreases the constitutive EGFR phosphorylation in H3255 cells and completely prevents EGF-mediated EGFR phosphorylation in A549 cells (Fig. 3.). In H1666 and H441, there is also constitutive EGFR phosphorylation, which can be further increased with EGF stimulation but is only minimally effected by gefitinib treatment. These studies were all performed with 1 μmol/L gefitinib, which is below the IC_{50} for H1666 and H441; with higher (10 μmol/L) gefitinib concentrations, EGFR phosphorylation is also inhibited in these cells, as we have shown previously (5). To determine whether EGFR may be constitutively phosphorylated in H1666 through an autocrine activation mechanism, we examined the media for the presence of TGF-α. In both H1666 and H441, we detect a small amount of TGF-α (mean, 19.8 and 29.3 pg/mL, respectively), but none was detected in H3255 or A549. This suggests that the constitutive activation of EGFR in H1666 and H441 may be due to an autocrine mechanism.

Our findings are somewhat different than those recently published by Lynch et al. (6), who observe no significant constitutive phosphorylation of EGFR^{L858R} compared with EGFR^{WT} when expressed in Cos-7 cells. Their findings demonstrate a greater and more sustained activation of EGFR^{L858R} compared with EGFR^{WT} after EGFR stimulation. One possibility for these differences is the presence of EGFR gene amplification in H3255. EGFR amplification alone could lead to ligand-independent activation that has been observed previously for both EGFR and ErbB-2 and may contribute to our findings (18, 19).

AKT but not ERK 1/2 Is Constitutively Active in H3255 Cells. There is constitutive EGFR activation in H3255 cells. We wished to determine whether pathways downstream of EGFR were also consti-

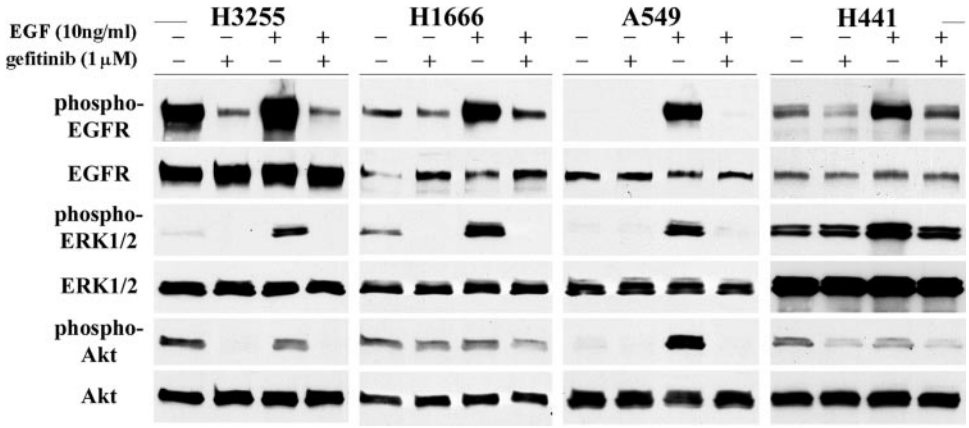

Fig. 3. Effects of gefitinib, EGF, and the combination on phosphorylation of EGFR and downstream AKT and ERK 1/2. NSCLC cells were serum starved for 24 hours and then grown in the presence or absence of 1 μmol/L gefitinib for 2 hours followed by EGF (10 ng/mL) stimulation for 15 minutes. Western blots are shown for phospho- and total EGFR, ERK 1/2, and AKT.

tutively active. AKT, but not ERK 1/2, is also constitutively phosphorylated in H3255 but not in A549 (Fig. 3). With EGF stimulation, phosphorylation of AKT increases substantially in A549 but does not increase and even decreases slightly in H3255. We have observed the latter finding multiple times, which may be due to activation of a negative feedback mechanism, and this is presently being explored. These findings suggest that the AKT pathway is preferentially activated by EGFR^{L858R}. In contrast to AKT, EGF stimulation increases ERK 1/2 phosphorylation in both H3255 and A549 cells. In both H1666 and H441, AKT is also constitutively phosphorylated but is minimally effected by either gefitinib or EGF and thus may be activated by other growth factor-dependent or -independent pathways. In H1666, there is constitutive phosphorylation of ERK 1/2 that can be further phosphorylated with the addition of EGF, findings that are similar in H3255, A549, and H441. Unlike AKT, phosphorylation of ERK 1/2 is completely inhibited by gefitinib treatment in H1666 cells, consistent with the effect of the drug on proliferation and cell cycle arrest in this cell line. There is no effect on ERK 1/2 phosphorylation by gefitinib in H441. To determine whether the constitutive ERK 1/2 phosphorylation is due to K-ras activation, we sequenced K-ras from H3255, H1666, and A549. No mutations in K-ras were found in either H3255 or H1666, but a codon 12 mutation was present in A549, consistent with prior observations (20). H441 is known to contain a K-ras codon 12 mutation.

Our findings have several potential clinical implications. If the preferential activation of the AKT pathway takes place consistently, then this suggests that targeted agents developed to inhibit this pathway may be therapeutically more effective than those designed to inhibit the ERK 1/2 pathway in patients whose tumors contain EGFR^{L858R}. Constitutive AKT activation has also been associated with resistance to chemotherapy and radiation in NSCLC cell lines (21). We have also demonstrated previously that the H3255 cell line is resistant to paclitaxel and docetaxel *in vitro* (7). It is thus possible that chemotherapy will be less effective in patients whose tumors contain EGFR^{L858R} than in those with a wild-type EGFR. Sequencing of EGFR from tumor specimens obtained from patients participating in large randomized trials of chemotherapy with gefitinib or placebo would be useful to help answer this hypothesis (22). Furthermore, combinations of chemotherapy with gefitinib or inhibitors of the AKT pathway should be tested *in vitro* in cell lines and *in vivo* in patients with EGFR^{L858R} mutations to determine whether these may be additive or synergistic.

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